Influence of Polymorphism in the Genes for the Interleukin (IL)-1 Receptor Antagonist and IL-1β on Tuberculosis

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Summary

Several lines of evidence suggest that host genetic factors controlling the immune response influence infection by Mycobacterium tuberculosis. The proinflammatory cytokine interleukin (IL)-1β and its antagonist, IL-1Ra (IL-1 receptor agonist), are strongly induced by M. tuberculosis and are encoded by polymorphic genes. The induction of both IL-1Rα mRNA and secreted protein by M. tuberculosis in IL-1Rα allele A2-positive (IL-1Rα A2+) healthy subjects was 1.9-fold higher than in IL-1Rα A2- subjects. The M. tuberculosis-induced expression of mRNA for IL-1β was higher in subjects of the IL-1β (+3953) A1+ haplotype (P = 0.04). The molar ratio of IL-1Rα/IL-1β induced by M. tuberculosis was markedly higher in IL-1Rα A2- individuals (P < 0.05), with minor overlap between the groups, reflecting linkage between the IL-1Rα A2 and IL-1β (+3953) A2 alleles. In M. tuberculosis-stimulated peripheral blood mononuclear cells, the addition of IL-4 increased IL-1α secretion, whereas interferon γ increased and IL-10 decreased IL-1β production, indicative of a differential influence on the IL-1Rα/IL-1β ratio by cytokines. In a study of 114 healthy purified protein derivative-reactive subjects and 89 patients with tuberculosis, the frequency of allelic variants at two positions (−511 and +3953) in the IL-1β and IL-1Rα genes did not differ between the groups. However, the proinflammatory IL-1Rα A2- /IL-1β (+3953) A1+ haplotype was unevenly distributed, being more common in patients with tuberculous pleurisy (92%) in comparison with healthy M. tuberculosis-sensitized control subjects or patients with other disease forms (57%, P = 0.028 and 56%, P = 0.024, respectively). Furthermore, the IL-1Rα A2- haplotype was associated with a reduced Mantoux response to purified protein derivative of M. tuberculosis: 60% of tuberculin-negative patients were of this type. Thus, the polymorphism at the IL-1 locus influences the cytokine response and may be a determinant of delayed-type hypersensitivity and disease expression in human tuberculosis.

Key words: interleukin 1 receptor • tuberculosis • susceptibility, disease • hypersensitivity, delayed • granuloma
TGF-β, IL-10, and, specifically, in the case of IL-1β, the IL-1 receptor antagonist (IL-1Ra), a pure antagonist of the IL-1 type 1 receptor (IL-1R1) (17). The coding for both IL-1β and the IL-1Ra gene are on chromosome 2q. Two allelic polymorphisms in the IL-1β gene at positions −511 and +3953 relative to the transcriptional start codon have been described (18, 19). Allele 1 of the +3953 polymorphism (IL-1β +3953 A1) is associated with moderately increased IL-1β production in response to LPS (19). The IL-1Ra gene is also polymorphic due to a variable number (2–6) of tandem repeats of 86 bp (VNTR) within its second intron (20). This polymorphism has been shown to be unambiguously functional at the level of secreted protein, as monocytes from individuals homo- or heterozygous for allele 2 (IL-1Ra A2, IL-1RN *2, 2 repeats) produce significantly more IL-1Ra in response to GM-CSF (21) and also have higher plasma levels (22). Serum IL-1Ra is known to be elevated in patients with disease expression in human tuberculosis.

In this study, we found that M. tuberculosis-induced IL-1Ra mRNA and protein secretion in healthy IL-1Ra A2 subjects was approximately twofold that of IL-1Ra A2 individuals. In addition, the molar ratio of IL-1Ra/IL-1β was strikingly higher in IL-1Ra A2 individuals. In M. tuberculosis-stimulated PBMC, the addition of IL-4 increased IL-1Ra secretion, whereas IFN-γ increased, and IL-10 decreased, IL-1β production, indicative of a differential influence on the IL-1Ra/IL-1β ratio by cytokines. In a pilot case-control analysis, the IL-1β and IL-1Ra allele frequencies were not different between patients with tuberculosis and purified protein derivative (PPD) skin test–reactive control subjects. However, the proinflammatory IL-1Ra A2/IL-1β (+3953) A1 haplotype was unevenly distributed, being more common in patients with pleural tuberculosis and less common in extrapulmonary disease. Furthermore, and consistent with the in vitro observations, the IL-1Ra A2 haplotype was associated with a reduced Mantoux response: 60% of tuberculin-nonreactive patients were of this type. Thus, the polymorphism at the IL-1 locus influences the cytokine response to, and may be a determinant of, delayed-type hypersensitivity (DTH) and disease expression in human tuberculosis.

Materials and Methods

Study Populations. For cell culture, healthy, PPD skin test-negative donors from the laboratory staff at Case Western Reserve University were bled and genotyped as below. In the pilot case-control analysis, a different population of 89 unselected patients and 114 control subjects who were Hindu, residents of London, and identified as being of Gujarati origin were recruited from Northwick Park Hospital, Harrow, England. The peak migration of Gujaratis to west London followed political change in East Africa in the decade 1970–1980. There is a high incidence of tuberculosis amongst Gujaratis in Harrow of ~128/100,000 (25), with an unusual excess of extrapulmonary disease in females. Within this community, 35–65% of marriages are prearranged, marriage to non-Gujarati Hindus is rare, and marriage to non-Hindus is exceptional (Patel, P., and R. J. Wilkinson, unpublished observations). 62% of subjects in this study were bacille Calmette-Guérin vaccinated. All 89 patients (average age 42.3 ± 1.7 yr; 56 females and 33 males) had culture- or biopsy-proven tuberculosis. All patients had free access to optimal medical care. The median duration of symptoms at diagnosis was 31 d (21 and 90 d being the 25th and 75th quartile values), thereby minimizing the effect of chronicity on clinical presentation. The definition of clinical phenotype was based on the International Classification of Disease 9 classification, and the overwhelming majority of patients were judged to have delayed postprimary (reactivation) disease. Patients known to be immunosuppressed (e.g., by HIV infection or corticosteroid therapy) were excluded. Mantoux testing was performed by the intradermal injection of one tuberculin unit of PPD (Evans Medical). The resultant diameter of transverse induration was recorded after 48 h. This low dose of tuberculin is routinely used in the United Kingdom to avoid necrotic reactions. All 114 nonconsanguineous (spouses of patients where possible) healthy controls (average age 42.9 ± 1.2 yr; 54 females and 60 males) were recruited from the tuberculosis contact clinic at the same hospital and had documented contact with tuberculosis (often multiple). All were PPD skin test–positive, asymptomatic, and had normal chest radiographs. 10/114 (8.7%) received chemophylaxis. These subjects were recruited between June 1995 and May 1998; in June 1998, all remained disease free. Ethical permission for this case-control analysis was obtained from the Harrow local research ethical committee (EC1646).

IL-1Ra and IL-1β G might have been investigated.

Materials and Methods

Study Populations. For cell culture, healthy, PPD skin test-negative donors from the laboratory staff at Case Western Reserve University were bled and genotyped as below. In the pilot case-control analysis, a different population of 89 unselected pa-

1 Abbreviations used in this paper: DTH, delayed-type hypersensitivity; PPD, purified protein derivative; rt, room temperature.

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C e l C u l t u r e . P B M C s were separated over a Ficoll (Phar- 
macia Biotech) gradient. Preliminary experiments established that 
conventional separation of monocytes by adherence to plastic, 
harvesting, and replating led to spontaneous release of IL-1Ra. 
T o reduce such activation, freshly isolated PBM C s were cultured 
at 2.5 × 10^6/ml in 24-well plates in RPMI 1640 (Biowhittaker) 
without antibiotics in the presence of 2% autologous serum. In 
each experiment, the number of monocytes present in PBM C s 
was determined by washing off nonadherent cells (×3) in a dupli-
cate well and then detaching the adherent cells using ice cold 
PBS and a cell scraper. M onocyte counts were generally ~10% of 
the total PBM C numbers. Preliminary experiments showed that 
IL-1Ra production under these conditions was detectable by 4 h 
and reached a plateau by 10–12 h, with no further significant in-
crease during the next 12 h. T here was no significant difference 
in production between experiments in which the nonadherent 
cells had been removed by washing and cells containing unsepa-
rated PBM C , indicating that the adherent cells were responsible 
for the IL-1Ra secretion. W e therefore collected, and froze at 
−70°C, PBM C supernates after 10 h of culture. In some cases, 
cell lysates were prepared by adding an equal volume of PBS and 
then freeze-thawing once. In this way we established that the ra-
tio of IL-1Ra secreted to that remaining in cell lysates was consist-
ently >10:1, irrespective of point of time, stimu-
lus, and genotype.

R eagents. M. tuberculosis H37R v was prepared and 
aliquoted as previously described (26). A liquots were 
vented for 15-20 min before use at an infection ratio of 0.1 or 1 
M. tuberculosis bacilli/1 PBM C (corresponding to ~1:1 and 10:1 
per monocyte). PPD of M. tuberculosis was the gift of Lederle 
Labs. (American Cyanamid Co.) and used at 0.1–100 µg/ml. 
R recombinant TGF-β, IL-4, and IFN-γ and the neutralizing an-
tibodies to IL-1β (mouse IgG2a), IL-6 (polyclonal goat IgG), 
TGF-β (polyclonal chicken IgY), and TNF-α (mouse IgG1), 
and appropriate isotype control antibodies were purchased from R & D 
Systems, Inc. All recombinant cytokines, PPD, M. tuberculosis, 
and neutralizing antibodies used were tested for endotoxin con-
tamination by the Limulus amebocyte assay (Biowhittaker) 
and were either free or contained very small levels (always <2 ng/ mg) 
of endotoxin.

C ytokine E LISA. M a xisorp (N unc, Inc.) plates were coated 
overnight at 4°C with 100 µl of the following coating antibodies 
in PBS: 2 µg/ml anti-human IL-1β mAb or 5 µg/ml of anti-
human IL-1Ra mAb (both from R & D Systems, Inc.). A fter 
washing in PBS/0.05% Tween 20 (×3), the plates were blocked 
for 1 h at room temperature (rt) using 300 µl 1% BSA/5% sucrose/ 
0.05% NaN₃ in PBS. A fter three further washes, duplicate 100-
µl samples and dilutions of standard cytokines were then incubated 
for 2 h at rt. A fter washing (×4), 100 µl of the following biotinyl-
lated detection antibodies were added in diluent (0.1% BSA, 
0.05% Tween 20 in TBS, pH 7.3): 100 ng/ml anti-human IL-1β 
antibody or 20 ng/ml anti-human IL-1Ra antibody (both from 
R & D Systems, Inc.). A fter 2 h at rt, the plates were washed (×5) 
and 100 µl streptavidin horseradish peroxidase (Jackson Immuno-
research) at 1:5,000 in diluent was added. A fter 20 min, six final 
washes were followed by the addition of 100 µl of 3,3',5,5'-tetra-
methylbenzidine hydrochloride solution in perborate (Sigma 
Chemical Co.) to each well. T he reaction was stopped by adding 
50 µl/well 0.5 N H₂SO₄, and the plates were read at 450 nm in 
an ELISA reader. T he sensitivity of each cytokine ELISA was as 
follows: IL-1β, <1 pg/ml and IL-1Ra, 0.05 ng/ml.

R ibonuclease P rotection A ssay. 5 × 10⁷ freshly isolated PBM C s 
were used to obtain ~5 × 10⁶ adherent cells. T his population of 
cells is up to 90% monocytes by cytostaining and is 99% viable 
(27). A fter resting overnight, the adherent cells were infected as 
above with M. tuberculosis at 1:1. A fter 4 h, the cells were har-
vested, and total RNA was extracted using guanidinium isothio-
 cyanate, CsCl₂ density gradient centrifugation, and ethanol pre-
cipitation. 2 µg of the resultant RNA was hybridized overnight 
according to the manufacturer’s instructions to a cocktail of 
[32P]UTP (Du Pont)-labeled complimentary RNA probes (Phar-
mingen) for IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-12 p40 and 
p35, TNF-α and -β, TGF-β₁₋₃, LT-β, and the housekeeping 
genes L32 and GAPDH at 56°C. Single-stranded RNA was di-
gested by incubation with RNase for 45 min at 37°C and the 
protected fragments reextracted by ethanol precipitation. T he 
products were electrophoresed on a 5% denaturing polyacryl-
amide gel; a negative control RNA and the unhybridized radio-
active probe were run in each experiment. T he gel was exposed 
overnight using a Biomard Gelsdor 10000. T he identity of the pro-
tected bands was confirmed by reference to the unhybridized 
probes and quantitated by reference to bands for the housekeep-
ging genes L32 and GAPDH.

C F U A ssay f or th e Intracellula r G rowth o f M. tuberculosis. T his 
assay was performed as previously described with minor modifi-
cations (26). In brief, adherent cells were plated in triplicate wells 
in 96-U microtiter plates (Corning Glass Works) and readded for 
2 h. C ells were infected with M. tuberculosis H37R v at 1:1, 10:1, 
and 100:1 (bacillus/cell) in 30% autologous serum. A fter 2 h, 
noningested bacteria were removed by washing gently (×3) with 
prewarmed RPMI 1640. E ach well then received RPMI 1640 
containing 2% autologous serum, and the plates were cultured in 
a humidified incubator at 37°C in the presence of 5% CO₂ for 
as long as 1 h (time 0 sample) up to 10 d. D uplicate wells contained 
2 µg/ml of neutralizing anti-IL-1Ra (goat IgG; R & D Systems, Inc.) 
or the same amount of isotype control antibody. A t the end of 
the culture period, supernates were aspirated, and the plates 
containing the infected adherent cells were frozen at −70°C. T o 
determine the number of intracellular bacteria in the CFU assay, 
the plates were thawed and cells lysed with 0.25% SDS in PBS 
for 12 min and then neutralized using 20% BSA. T he lysates 
were then 10-fold serially diluted with 7H9 broth (Difco Labs., Inc.), 
and three 10-µl aliquots of each dilution were plated on Middel-
brook 7H10 agar (Difco Labs., Inc.) to obtain CFUs. T he CFUs at 
de the end of this culture period, the number of CFUs in each of 
the three replicate spots was enumerated for at least two consecutive 
dilutions using a stereomicroscope and averaged. U sing this tech-
nique, extracellular growth of mycobacteria as assessed by culture 
of the supernates is consistently >1 log lower than intracellular 
growth (26). T he rate of intracellular growth expressed as dou-
bling time was determined by reference to the logarithmic growth 
from the cultures.

Statistical A nalysis. V alues throughout are quoted or shown as 
the mean ± SE. N ormally distributed variables were analyzed 
by paired or unpaired t test. P values reflect two-tailed values of t. 
U npaired nonparametric variables were analyzed by the M ann-
Whitney U test. Contingency analysis was performed using Fisher’s 
extact test of probability.

R e s u lts

P olymorphism in the IL-1Ra gene A ssociates with the Stimu-
lated Production of IL-1Ra. F irst, we examined the M. tuber-
culos is-stimulated production of IL-1Ra by culture of 2.5 ×
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10^6 PBM C s for 10 h. Culture supernates were assayed for IL-1Ra content, and the results were normalized to the number of monocytes in culture. The relationship between polymorphism in IL-1RN in 17 donors homozygous for the A1 allele (IL-1Ra A2^-) and 16 donors at least heterozygous for A2 (3 A2/A2, 13 A1/A2; IL-1Ra A2^+) and the M. tuberculosis-induced secretion of IL-1Ra was determined. The other alleles of IL-1RN were very rare and therefore could not be assessed. The M. tuberculosis-stimulated IL-1Ra A2 response to A1/A2 homozygotes did not differ from A1/A2 (data not shown), confirming the previous finding that IL-1Ra A2 is codominant (21). The unstimulated production of IL-1Ra A2 was slightly, but not significantly, higher in the IL-1Ra A2^+ group (Fig. 1 A). Stimulation by M. tuberculosis (0.1 and 1:1 bacillus/cell) caused a dose-dependent increase in IL-1Ra secretion irrespective of genotype. However, the median response of the IL-1Ra A2^+ group was 1.9 times greater at both doses of M. tuberculosis tested (P = 0.02 at 1:1). In a subset of 16 healthy subjects, the dose response of IL-1Ra induction to PPD was also determined (Fig. 1 B). Although IL-1Ra A2^- individuals showed a dose-dependent increase in IL-1Ra secretion, this did not become statistically significant until the dose of PPD was 100 μg/ml. The response of IL-1Ra A2^+ individuals was 2.1–3.6 times higher, depending on the dose. In contrast, induction of IL-1Ra in IL-1Ra A2^+ donors was significant at 1 μg/ml. Thus, IL-1Ra A2^- donors appeared more sensitive to PPD stimulation. The median production of IL-1Ra in response to LPS (10 μg/ml) was also 1.82 times greater in the IL-1Ra A2^+ donors (6.6 ± 1.3 vs. 3.6 ± 0.5 ng/ml/10^6 monocytes, P = 0.012).

Relationship between Polymorphisms and the Production of IL-1β. We next determined the level of IL-1β in the same culture supernates used for the analysis of IL-1Ra. In contrast to the IL-1Ra polymorphism, the two polymorphisms in the IL-1β gene did not correlate with the M. tuberculosis-stimulated production of IL-1β to the same extent. The median M. tuberculosis (at 1:1)-stimulated production of IL-1β in subjects positive for the −511 A2 (n = 20) was 635 ± 119 pg/ml and 404 ± 261 pg/ml in A1/A1 homozygotes (n = 8). The corresponding figures for the +3953 polymorphism were 404 ± 84 pg/ml (A2^+, n = 12) and 643 ± 171 pg/ml (A1/A1 homozygotes, n = 16). IL-1β production did tend to be higher in IL-1Ra A2^- subjects, but only significantly so in response to M. tuberculosis at 0.1% (P = 0.01) (data not shown).

As a pure antagonist of IL-1, IL-1Ra competes for occupancy of IL-1RI, and it has been estimated that IL-1Ra needs to be present in a large molar excess (25–50 ×) to antagonize IL-1β significantly (28). Therefore, the ratio of IL-1Ra/IL-1β is likely to be more relevant to regulation of the inflammatory response than the absolute value of either cytokine. The molar ratio of IL-1Ra/IL-1β was therefore calculated for each supernate and was significantly higher in IL-1Ra A2^+ individuals (P < 0.05) in response to doses of both M. tuberculosis and PPD at 1, 10, and 100 μg/ml (Fig. 2 B), in some cases with only minor overlap between the groups. By contrast, the response to LPS did not differ significantly between the groups. Fig. 2 B shows that the highest ratios likely resulted in antagonism of the IL-1β response to PPD and M. tuberculosis stimulation (especially at lower doses likely to be relevant to M. tuberculosis-infected foci) were observed in the majority of IL-1Ra A2^+ individuals but only in a minority of IL-1Ra A2^- subjects. The bulk of the experiments were performed using attenuated M. tuberculosis H37Rv in three donors (one A1/A1 and two A1/A2) was also performed. The level of each cytokine was very similar, such that at an infection multiplicity of 1:1 the IL-1Ra/IL-1β ratio when stimulated by H37Rv was 4.1, 16.8, and 12.6 for the three donors and 6.2, 17.3, and 8.0, respectively when stimulated by H37Rv. We thus have no reason to suspect that the findings using M. tuberculosis H37Rv would not apply to virulent clinical isolates.
Cytokine Gene Expression by Ribonuclease Protection Assay. We next sought to investigate association between the polymorphisms and the expression of mRNA. Ribonuclease protection assay was performed on RNA from 13 donors, all of different genotypes. The spontaneous expression of IL-1Ra and IL-1β transcript was low. There was no constitutive expression of any other monocyte cytokine, indicating that this low expression was unlikely to have been due to a non-specific effect of cellular activation during isolation. Within 1 h, M. tuberculosis induced IL-1α gene expression in all individuals irrespective of genotype, together with the mRNAs for IL-1β and TNF-α and followed slightly later (2 h) by IL-1α and IL-6. At hour 4, there was higher induction of IL-1α in the IL-1Ra A2+ subjects consistent with the protein data, although the difference was not statistically significant (Table I). The IL-1β (+3953) allele A2+ was associated with significantly lower production of IL-1β transcript (P = 0.04). Taken together, we interpret these observations to indicate that the alleles are associated with differences in transcription, but the dissociation between induction and secretion, particularly in the case of IL-1β, indicates that post-transcriptional mechanisms also influence cytokine secretion.

Effect of Monocyte Cytokines on the Production of IL-1Ra in Response to M. tuberculosis. The results so far showed that in response to M. tuberculosis or its PPD, IL-1Ra a gene expression is induced within 1 h, large quantities of protein are secreted within 10 h, and differences between individuals could be related to their genotypes. However, an indirect modulating influence of M. tuberculosis via increased translation of preexisting IL-1Ra mRNA or an effect of other cytokines (such as TGF-β, TNF-α, IL-1β, and IL-6) produced by monocytes early in response to infection is also possible. We investigated this possibility by assessing the ability of antibodies known to neutralize the biological effects of TGF-β, TNF-α, IL-1β, and IL-6 on M. tuberculosis-stimulated production of IL-1Ra. Control wells received isotype-matched antibodies. No consistent effect on constitutive or stimulated IL-1Ra secretion was seen, irrespective of genotype, cytokine, or dose of antibody used (up to 1,000-fold the ED50 concentrations). TGF-β modulates the human response to tuberculosis (29, 30) and has also been reported to increase IL-1Ra in some (31) but not all studies (32). We therefore also evaluated the effect of TGF-β (0.1-10 ng/ml) on both M. tuberculosis-stimulated and -unstimulated IL-1Ra a production in 12 individuals (6 IL-1Ra A2+ and 6 IL-1Ra A2+). No significant enhancement of the early secretion of IL-1Ra was seen (data not shown). However, rIL-10 (0.1-10 ng/ml) caused a significant dose-dependent increase in the M. tuberculosis-stimulated IL-1Ra a/IL-1β ratio in IL-1Ra A2+ and IL-1Ra A2+ donors at all doses tested (P < 0.02), an effect largely due to the suppression of IL-1β production (Fig. 3A). The addition of rhIL-6, however, caused no significant change in the IL-1Ra a/IL-1β ratio in either group.

Effect of T Cell Cytokines on the Production of IL-1Ra a and IL-1β in Response to M. tuberculosis. It has also been shown that the lymphocyte production of IFN-γ and IL-4 can differentially modulate IL-1β and IL-1Ra a production (33). Our coculture system excluded the possibility of an obscuring effect of T cell cytokines by the sole use of PBMCs from PPD–individuals and a short culture duration. In fact, the production of IFN-γ was negligible in the M. tuberculosis-stimulated cultures (20 pg/ml) from these subjects. To investigate the possibility that T cell cytokines modulate M. tuberculosis-induced IL-1Ra a and IL-1β secretion, rhIFN-γ or rhIL-4 were added (0.1-10 ng/ml) to cultures. IL-4 caused a dose-dependent increase in both unstimulated and M. tuberculosis-stimulated IL-1Ra a production, which was most significant in the M. tuberculosis-stimulated

<table>
<thead>
<tr>
<th>Haplotype Number</th>
<th>Fold induction*</th>
<th>R range</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1Ra a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>5</td>
<td>5.7</td>
</tr>
<tr>
<td>A2+</td>
<td>8</td>
<td>10.0</td>
</tr>
<tr>
<td>IL-1β (-511)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>3</td>
<td>25.9</td>
</tr>
<tr>
<td>A2+</td>
<td>10</td>
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<tr>
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</tr>
<tr>
<td>A2+</td>
<td>6</td>
<td>29.3</td>
</tr>
</tbody>
</table>

*P values were 0.30 for IL-1Ra a, 0.14 for IL-1β (-511), and 0.04 for IL-1β (+3953).
IL-1Ra A2+ group (P = 0.002 at 10 ng/ml). Furthermore, IL-4 also significantly decreased IL-1β production in M. tuberculosis-stimulated cells from both genotypes (P < 0.05 at 10 ng/ml). By comparison, IFN-γ led to a dose-dependent increase in M. tuberculosis-stimulated IL-1β production that was most marked in the IL-1Ra A2+ group (P = 0.052 at 10 ng/ml). Thus, IFN-γ tended to increase IL-1β production in M. tuberculosis-stimulated cells without affecting IL-1Ra production, whereas IL-4 increased IL-1Ra production irrespective of genotype and also depressed IL-1β secretion. This differential effect was reflected in the mean M. tuberculosis-stimulated IL-1Ra/IL-1β ratio, which increased in response to IL-4 even at the lowest dose of 0.1 ng/ml (P < 0.01, both groups combined). By comparison, higher doses of IFN-γ (1–10 ng/ml) were required to reduce the IL-1Ra/IL-1β ratio significantly (Fig. 3 B).

Relationship Between Polymorphism in IL-1Ra and the Intraacellular Growth of M. tuberculosis. We next investigated the effect of IL-1Ra a polymorphism on the rate of intracellular replication of M. tuberculosis. Monocytes from 22 donors (12 IL-1Ra a A2− and 10 IL-1Ra a A2+) were infected with M. tuberculosis at various multiplicities (1:1, 10:1, and 100:1 bacillus/cell) and then cultured in vitro for up to 240 h. Cell lysates were set up for M. tuberculosis CFU assay at 0, 24, 96, 168, and 240 h. Although there was interindividual variation in the establishment of initial infection, there was no significant difference between the IL-1Ra a A2− and IL-1Ra a A2+ groups. Logarithmic growth was established in 8 donors. The remainder showed either minimal or linear intracellular growth of mycobacteria only, with no difference between IL-1Ra a A2− and IL-1Ra a A2+ donors. In those donors in whom logarithmic growth did occur (5 IL-1Ra a A2− and 3 IL-1Ra a A2+), the doubling time of M. tuberculosis was estimated from the growth curve. Data from these individuals is shown in Table II. Intra- and interindividual differences did not appear to be related to the presence or absence of polymorphism. The doubling times were estimated from the logarithmic growth curve in each case. The numbers in parentheses show the IL-1Ra level in ng/ml/100,000 monocytes for the same individual at the corresponding multiplicity of infection.

Table II. Lack of Relationship between IL-1Ra Polymorphism and the Intracellular Growth of M. tuberculosis In Vitro

<table>
<thead>
<tr>
<th>Donor</th>
<th>Genotype</th>
<th>Culture duration</th>
<th>Doubles at various multiplicities of infection by M. tuberculosis (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>A1/A1</td>
<td>96</td>
<td>24 (2.47)</td>
</tr>
<tr>
<td>2</td>
<td>A1/A1</td>
<td>168</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>A1/A1</td>
<td>240</td>
<td>39 (2.56)</td>
</tr>
<tr>
<td>4</td>
<td>A1/A1</td>
<td>240</td>
<td>38 (1.45)</td>
</tr>
<tr>
<td>5</td>
<td>A1/A1</td>
<td>240</td>
<td>42</td>
</tr>
<tr>
<td>6</td>
<td>A1/A2</td>
<td>96</td>
<td>27 (10.65)</td>
</tr>
<tr>
<td>7</td>
<td>A1/A2</td>
<td>168</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>A1/A2</td>
<td>240</td>
<td>53 (10.67)</td>
</tr>
</tbody>
</table>

Table II. Lack of Relationship between IL-1Ra Polymorphism and the Intracellular Growth of M. tuberculosis In Vitro

Donors in whom logarithmic growth did occur (5 IL-1Ra a A2− and 3 IL-1Ra a A2+), the doubling time of M. tuberculosis was estimated from the growth curve. Data from these individuals is shown in Table II. Intra- and interindividual differences did not appear to be related to the presence or absence of polymorphism. The doubling times were estimated from the logarithmic growth curve in each case. The numbers in parentheses show the IL-1Ra level in ng/ml/100,000 monocytes for the same individual at the corresponding multiplicity of infection.

—, Not tested.
Allele 1 was in linkage disequilibrium with IL-1β, and were in Hardy-Weinberg equilibrium. The IL-1β allele, distinct and has a high incidence of tuberculosis with an excess of extrapulmonary forms. Individual alleles at each locus were in Hardy-Weinberg equilibrium. The IL-1β allele 1 was in linkage disequilibrium with IL-1β (−511) allele 2 and vice-versa (P < 0.03). In addition, there was weaker linkage between IL-1R α A2 and IL-1β (+3953) A2. No allele or genotype, singly or in combination, was associated with an increased risk of tuberculosis (Table III). We concluded that, in this population, these polymorphisms have little effect on susceptibility to tuberculosis per se.

The in vitro data indicated that the IL-1R α A2+/IL-1β (+3953) A1+ haplotype was associated with low IL-1R α protein and gene expression and higher corresponding IL-1β values in vitro, implying a proinflammatory phenotype. This haplotype was more common in pleural disease, a form in which DTH is thought to be high, and was also associated with a moderately greater reaction to PPD in vivo. P values were calculated relative to the control group by Fisher's exact test of probability, except for the comparison of median Mantoux diameter within the patient group, which was performed by the Mann-Whitney U test.

**Discussion**

We have investigated the effect of polymorphisms in the IL-1β and IL-1R α genes on M. tuberculosis-stimulated cyto-

### Table III. IL-1Ra and IL-1β A allele and G genotype frequencies in T tuberculosis Patients and Tuberculin-reactive Healthy Control Subjects

<table>
<thead>
<tr>
<th>Gene Position</th>
<th>Genotype or allele frequency</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1R α N/A</td>
<td>A1 0.713, 0.719</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2 0.236, 0.241</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3 0.045, 0.039</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4 0.006, 0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1/A1 51 (57%), 65 (57%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1/A2 21 (24%), 30 (26%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2/A2 9 (10%), 10 (9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Others* 8 (9%), 9 (8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β −511</td>
<td>A1 0.438, 0.404</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2 0.562, 0.596</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1/A1 20 (22%), 18 (16%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1/A2 38 (43%), 56 (49%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2/A2 31 (35%), 40 (35%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β +3953</td>
<td>A1 0.837, 0.794</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2 0.163, 0.206</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1/A1 64 (72%), 76 (67%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1/A2 21 (24%), 29 (25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2/A2 4 (4%), 9 (8%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers in italics represent allele frequencies in each group for each locus. The numbers in plain text represent the number (accompanied by the percentage) of individuals with a given genotype. There are no significant differences in allele or genotype frequencies between patients (n = 89) and healthy control subjects (n = 114) at any of the three loci.

*Patients three A1/A3, one A3/A1, one A1/A4, and four A2/A3; controls four A1/A3 and five A2/A3.

### Table IV. Relationship between IL-1R α/IL-1β Haplotype and Disease Phenotype

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-1R α A2+/IL-1β (+3953) A1+ haplotypes*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T total controls</td>
<td>65 (57%)</td>
<td>49 (43%)</td>
</tr>
<tr>
<td>T total patients</td>
<td>54 (61%)</td>
<td>35 (39%)</td>
</tr>
<tr>
<td>Pleural</td>
<td>11 (92%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>16 (62%)</td>
<td>10 (38%)</td>
</tr>
<tr>
<td>Miliary</td>
<td>6 (60%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>Lymphadenopathic</td>
<td>12 (60%)</td>
<td>8 (40%)</td>
</tr>
<tr>
<td>Extrapulmonary</td>
<td>9 (43%)</td>
<td>12 (57%)</td>
</tr>
<tr>
<td>Median Mantoux/mm</td>
<td>15.5 ± 1.4</td>
<td>11.5 ± 1.8</td>
</tr>
</tbody>
</table>

The number of patients with varying disease forms bearing the IL-1R α A2+/IL-1β (+3953) A1+ haplotype is compared to the number bearing other combinations. This haplotype was associated with low IL-1R α protein and gene expression and higher corresponding IL-1β values in vitro, implying a proinflammatory phenotype. This haplotype was more common in pleural disease, a form in which DTH is thought to be high, and was also associated with a moderately greater reaction to PPD in vivo. P values were calculated relative to the control group by Fisher's exact test of probability, except for the comparison of median Mantoux diameter within the patient group, which was performed by the Mann-Whitney U test.

*Controls: 40 IL-1R α A2+/IL-1β (+3953) A1+, 5 IL-1R α A2+/IL-1β (+3953) A1+; and 4 IL-1R α A2+/IL-1β (+3953) A1+. Patients: 31 IL-1R α A2+/IL-1β (+3953) A1+, 2 IL-1R α A2+/IL-1β (+3953) A1+, and 2 IL-1R α A2+/IL-1β (+3953) A1+.

10 osteomyelitis, 2 adrenal, 2 ileocaecal, 2 psoas, 2 synovial, 1 thigh abscess, 1 pericardial, and 1 peritoneal.

### Discussion

We have investigated the effect of polymorphisms in the IL-1β and IL-1R α genes on M. tuberculosis-stimulated cyto-
affected by antibody neutralization of IL-1β, IL-6, TGF-β, and TNF-α, suggesting that M. tuberculosi's or its products induce the early production of large quantities of IL-1Ra by a direct mechanism.

IL-1β is involved in the early recruitment of inflammatory cells to M. tuberculosis- or PPD-induced granulomas (37–41). Submaximal occupancy of IL-1Rls can mediate the full biological effects of IL-1β, and as a consequence, it has been postulated that IL-1Ra needs to be present in a large molar excess in order to exert its antagonism (28). In tuberculosis, this condition would be best fulfilled in IL-1R a2 subjects (Fig. 2); the IL-1R a2 allele was associated with reduced DTH (Fig. 4) and was lower in frequency in patients with pleural tuberculosis, consistent with the in vitro data and suggestive of biological significance. Anti-gen-specific lymphocytes are also necessary for the DTH reaction to proceed. In our experiments, IL-4 increased IL-1Ra a1 secretion, particularly in stimulated cultures from IL-1R a2 subjects (Fig. 3B). The production of IL-4 in tuberculosis has been best demonstrated in T cell clones (42), but one study has also documented small amounts of antigen-specific secretion of IL-4 by PBMCs (43). As cell-associated IL-4 is a stimulus for IL-1Ra, there is the possibility that relatively small amounts of IL-4 may greatly affect the IL-1Ra a1 response (33). IFN-γ decreased and IL-10 increased the IL-1Ra a1/IL-1β ratio mainly through an effect on IL-1β secretion. Both IFN-γ and IL-10 are produced by PBMCs and at disease sites in patients with tuberculosis (29, 44, 45). Our data therefore suggests that the polymorphism in the IL-1R a1 gene may exert regulatory influence on cytokine circuits beyond its direct effect on IL-1Ra production.

There is both epidemiological and experimental evidence of a dissociation between DTH and protection from tuberculosis (46, 47). Our finding that IL-1R a appears to influence DTH with minimal effect on either the intracellular growth of M. tuberculosis in vitro or disease susceptibility in the case-control study further suggests a basis for the dissociation between DTH and susceptibility. In addition to disease susceptibility, the degree of cutaneous reactivity to PPD after bacille Calmette-Guérin vaccination in both mono- and dizygotic twins and in siblings is also heritable (48, 49). Our data therefore suggests that the polymorphism in the IL-1R a1 gene may exert regulatory influence on cytokine circuits beyond its direct effect.

Our study of IL-1RN gene expression indicates the early induction by M. tuberculosis of its mRNA together with IL-1β, IL-1α, TNF-α, and IL-6. Although IL-1R a2 was associated with an increased induction of the IL-1RN gene, the exact mechanism of increased IL-1Ra production requires further elucidation. Whereas the fold induction of IL-1β mRNA was higher than that of IL-1R a and could also be related to both IL-1β polymorphisms, the amount of secreted IL-1β protein was much less. In addition, the IL-1β polymorphisms could not so readily be related to protein secretion. This observation is consistent with other data (36) and indicates a dominant influence of both posttranscriptional and posttranslational events on the secretion of IL-1β. Many cytokines can upregulate IL-1R a expression in vitro (17). The production of IL-1R a, however, was un-
We propose that the early recruitment and activation of in-genes can influence disease phenotype in tuberculosis (52). Expression supports the hitherto unproven concept that host genes can influence disease phenotype in tuberculosis (52). We propose that the early recruitment and activation of inflammatory cells by IL-1β to foci of tuberculous infection is in turn downregulated by IL-1R a that, under polymorphic host control, acts to limit the resultant DTH. This hypothesis could be readily tested in IL-1 and IL-1Ra gene knockout mice (53, 54). Reduction of DTH by targeted immunotherapy with either IL-1Ra or other engineered antagonists of IL-1Ra (55) may also be a possible approach to modulation of immunopathologic cytokine circuits in tuberculosis.

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